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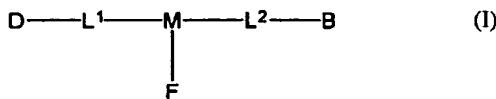
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(54) Title: SITE-SPECIFIC LABELLING OF PROTEINS USING CYANINE DYE REPORTERS



WO 2004/011556

(57) Abstract: Disclosed are compounds of formula (I):in which D is a dye selected from a cyanine dye or a derivative thereof; B is an affinity tag; F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group; M is a group adapted for attaching to F; and L¹ and L² each independently comprise a group containing from 1 to 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from -NR'-, -O-, -CH=CH-, -CO-NH- and phenylenyl groups, where R' is selected from hydrogen and C₁ - C₄ alkyl. The invention also relates to methods that afford direct attachment of the cyanine dye reporter group to either the N-terminus or C-terminus of a synthetic or recombinant peptide or protein, and their derivatives, in a site-specific manner, coupled with purification of the resultant labelled molecule.

Site-specific Labelling of Proteins using Cyanine Dye Reporters

The present invention relates to reagents and methods for site-specific labelling of proteins using cyanine dyes as reporter molecules. In particular,

- 5 the invention relates to new cyanine dye derivatives containing thioester activated groups and groups reactive with target molecules containing or derivatised to contain a thioester reactive moiety.

There is increasing interest in, and demand for, fluorescent reporters
10 for use in the labelling and detection of biomolecules. Cyanine and related dyes such as rigidised cyanine dyes and squaraines offer a number of advantages over other fluorescent dye reagents and they are finding widespread use as fluorescent labels in such diverse areas as sequencing, microarrays, flow cytometry and proteomics. For example, US 5569587

- 15 (Waggoner et al) discloses water soluble cyanine dye derivatives that possess reactive groups suitable for reaction with target molecules that contain, or are derivatised to contain, -OH, -NH₂, or -SH groups. The cyanine dyes are characterised by having very high extinction coefficients and favourable quantum yields. In addition, cyanine dyes possess good photostability and
20 are not readily photobleached.

In many applications there is a need to form a permanent link, in the form of a covalent bond, between a fluorescent labelling dye and a target molecule such as a protein. The chemistry of peptide and protein labelling is
25 well documented and a wide range of labelling reagents are now commercially available. For a review and examples of protein labelling using fluorescent labelling reagents, see "Non-Radioactive Labelling, a Practical Introduction", Garman, A.J. Academic Press, 1997; "Handbook of Fluorescent Probes and Research Chemicals", Haugland, R.P., Molecular Probes Inc., 1992).

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Site-specific incorporation of a fluorescent label into a protein or peptide may be of considerable benefit in certain biochemical and biophysical studies, for example fluorescence resonance energy transfer, and protein

structure and function studies. One method for the site-specific attachment of a fluorescent label into a target polypeptide utilises the native chemical ligation reaction. According to this procedure, an unprotected peptide fragment containing an N-terminal cysteine residue and a second unprotected peptide fragment containing an α -thioester group are chemoselectively ligated together at physiological pH, irrespective of their primary sequences, to generate an amide bond at the ligation site. For examples, see reviews by Cotton, G.J. and Muir T.W., Chem. Biol., (1999), 6, R247-260; Giriati, I., Muir, T.W. and Perler, F.B., Genetic Engineering, (2001), 23, 171-199; Muir, T.W., Syn. Lett., (2001), 6, 733-740.

Tolbert, T.J. and Wong, C-H. (Angew. Chem. Int. Ed., (2002), 41, 2171-2174) describe the preparation of fluorescein and biotin thioester derivatives and the reaction of these with N-terminal cysteine-containing recombinant proteins. Schuler, B. and Pannell, L.K. (Bioconjugate Chemistry, published on line, 18 July 2002) reported the preparation of a benzyl thioester of Cy5TM and subsequent reaction with a synthetic polypeptide containing an N-terminal cysteine residue.

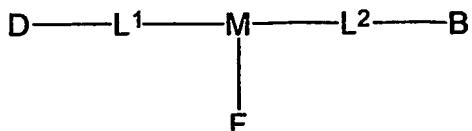
However, there are no reports describing thioester derivatives of cyanine dyes in which the reporter is also linked covalently to an affinity tag. Use of such a reagent in reactions involving site specific labelling of proteins and peptides will be advantageous for subsequent separation and purification of the fluorescent dye-labelled target. The present invention therefore provides new cyanine dye reagents and methods that afford direct attachment of the cyanine dye reporter to either the N-terminus or C-terminus of a synthetic or recombinant peptide or protein and their derivatives, in a site-specific manner, coupled with purification of the resultant labelled molecule.

According to one aspect of the present invention, there is provided a compound comprising a cyanine dye or derivative thereof containing at least one target bonding group selected from a carboxylic acid thioester group or a

group suitable for covalent reaction with a thioester, characterised in that said compound includes an affinity tag covalently bound thereto.

Suitably, the compound is of formula (I):

5



10

(I)

wherein:

D is a dye selected from a cyanine dye or a derivative thereof;

B is an affinity tag;

15 F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group;

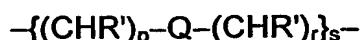
M is a group adapted for attaching to F; and

L¹ and L² each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl

20 groups, where R' is selected from hydrogen and C₁ – C₄ alkyl.

Suitably, there are 2 to 30 atoms in each of L¹ and L², preferably, 6 to 20 atoms.

25 Preferably, L¹ and L² are independently selected from the group:



where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and

30 –CO–NH–; R' is hydrogen or C₁ – C₄ alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

Particularly preferred Q is selected from: -CHR'-, -O- and -CO-NH-, where R' is hereinbefore defined.

In one embodiment L² is a cleavable linker and may additionally include
5 group P which may be suitably selected from a chemically-cleavable group, an enzyme-cleavable group, or a photochemically-cleavable group. Suitable chemically cleavable groups include carbamate esters and carboxylate esters, which are both cleaved under basic conditions. Suitable enzyme cleavable groups may be selected from groups such as ester, amide and phospho-
10 diester groups. Such groups are substrates for, and are hydrolysed by hydrolases, such as proteases, esterases and phospho-diesterases. Suitable photocleavable groups P for use in the compound of formula (I) may contain the 4,5-dialkoxy-2-nitrobenzyl alcohol linker (Holmes, C.P., and Jones, D.G., J.Org.Chem., (1995), 60, 2318-2319) or phenacyl linkers (Wang, S.,
15 J.Org.Chem., (1976), 41, 3258-3261). These groups undergo efficient photoreaction upon 300nm illumination, resulting in the rapid cleavage of the dye molecule or dye-labelled protein from the affinity tag.

Suitably, the group M may be any suitable functional group adapted for
20 attaching the target bonding group F. Preferably, M is selected from:



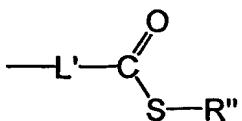
wherein R' is hereinbefore defined.

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Suitable affinity tags may be selected from biotin, desthiobiotin and metal chelating ligands such as his-tag and iminodiacetic acid, nitrilotriacetic acid and the like. Preferred affinity tags are selected from biotin and desthiobiotin.

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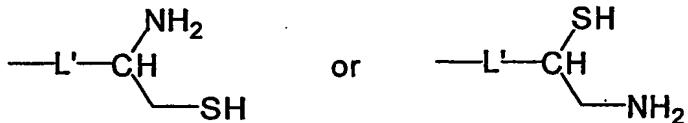
In one embodiment of the present invention, the target bonding group F is a carboxylic acid thioester of formula:



- 5 wherein L' is a bond or is a group containing from 1 – 30 linked atoms selected from carbon atoms and optionally one or more groups selected from –NH–, –O– and –CO–NH–; and R" is C₁ – C₄ alkyl, C₆ – C₁₀ aryl, or C₇ – C₁₅ aralkyl, which may be optionally substituted with sulphonate; or is the group –(CH₂)₂–CONH₂. In the case where L' is a bond, the target bonding group F
 10 is attached directly to group M.

In an alternative embodiment, the target bonding group F is a 1,2-aminothiol group of formula:

15



wherein L' is hereinbefore defined.

- 20 Thus, the present invention provides fluorescent labelling reagents comprising a cyanine dye or derivative thereof, that are modified by incorporating a target bonding group and an affinity tag into the molecule. The target bonding group may be selected from a carboxylic acid thioester group or a 1,2-aminothiol group. Where the target bonding group is a
 25 thioester group, it is selectively reactive with a 1,2-aminothiol group on a target molecule, suitably a protein or peptide, or a derivative thereof. In the alternative, the cyanine dye may contain a 1,2-aminothiol group for reaction with a thioester group on the target. The incorporation of a reactive thioester or, alternatively, a 1,2-aminothiol functionality into the chemical structure of
 30 the reporter molecule enables the target molecule to be directly labelled in a convenient one step process. According to the methods of the invention, labelling of peptides and proteins is site-specific, irrespective of the composition of the primary sequence. By generating the target primary

sequence with either an N-terminal cysteine or a thioester functionality, site-specific labelling can be achieved directly, by incubating the target with the appropriate derivative of the cyanine dye, suitably, the thioester and 1,2-aminothiol derivatives respectively. Furthermore, inclusion of an affinity tag in
 5 the labelling reagent allows subsequent purification of the fluorescent dye labelled protein or peptide.

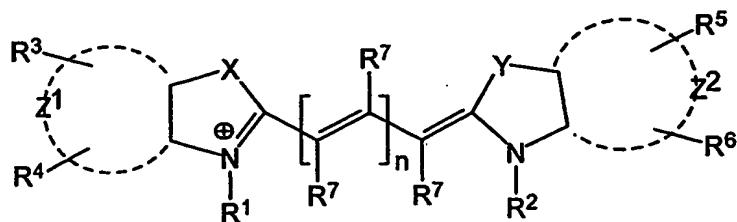
Suitably, the cyanine dye or cyanine dye derivative may be selected from cyanine dyes, rigidised cyanine dyes and squaraine dyes, provided that
 10 the dye incorporates at least one carboxylic acid thioester group, or a group suitable for covalent reaction with a thioester. Table 1 shows some examples of cyanine dyes, having particular excitation (Abs) and emission (Em) characteristics.

15 Table 1

<u>Dye</u>	<u>Fluorescence Colour</u>	<u>Abs (nm)</u>	<u>Em (nm)</u>
Cy2	Green	489	506
Cy3	Orange	550	570
Cy3.5	Scarlet	581	596
Cy5	Far red	649	670
Cy5.5	Near-IR	675	694
Cy7	Near-IR	743	767

In one embodiment according to the first aspect, the compound has the formula (II):

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(II)

wherein:

groups R³ and R⁴ are attached to the Z¹ ring structure and groups R⁵ and R⁶ are attached to the Z² ring structure;

n is an integer from 1 to 3;

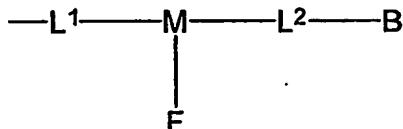
Z¹ and Z² independently represent the atoms necessary to complete one ring

5 or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

X and Y are the same or different and are selected from: >CR⁸R⁹, oxygen, sulphur, -CH=CH-, >N-W wherein N is nitrogen and W is selected from

10 hydrogen and the group R¹⁰;

at least one of groups R¹, R², R³, R⁴, R⁵, R⁶, R⁸, R⁹ and R¹⁰ is the group:

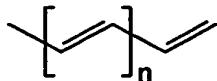


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where B, F, M, L¹ and L² are hereinbefore defined;

groups R⁷ are independently selected from hydrogen and C₁ – C₄ alkyl which may be unsubstituted or substituted with aryl, or two or more of R⁷ together with the group:

20



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form a hydrocarbon ring system substituted with R⁷ and which may optionally contain a heteroatom selected from -O-, -S- or >NR⁷, wherein R⁷ and n are

hereinbefore defined;

remaining groups R³, R⁴, R⁵ and R⁶ are independently selected from the group consisting of hydrogen, halogen, amide, cyano, nitro, mono- or di-C₁ – C₆ alkyl-substituted amino, carbonyl, carboxyl, C₁ – C₆ alkyl, C₁ – C₆ alkoxy, aryl, heteroaryl, aralkyl and the group -(CH₂)_m-Y where Y is selected from

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sulphonate, sulphate, phosphonate, phosphate and quaternary ammonium and m is zero or an integer from 1 to 6;

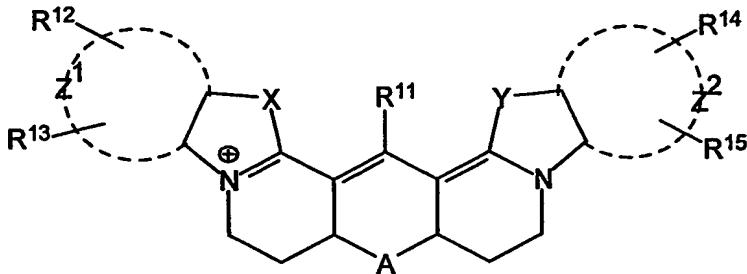
remaining groups R⁸, R⁹ and R¹⁰ are independently C₁ – C₆ alkyl; and

remaining groups R¹ and R² are independently selected from hydrogen, C₁ – C₁₀ alkyl, the group –(CH₂)_m–Y wherein Y and m are hereinbefore defined, and benzyl which may be unsubstituted or substituted by up to two nitro groups.

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In a second embodiment according to the first aspect, the compound has the formula (III):

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(III)

wherein

groups R¹², R¹³, R¹⁴ and R¹⁵ are attached to the rings containing X and Y or, optionally are attached to atoms of the Z¹ and Z² ring structures;

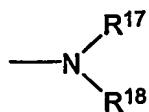
20 Z¹ and Z² independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

X and Y are the same or different and are selected from: >CR⁸R⁹, oxygen,

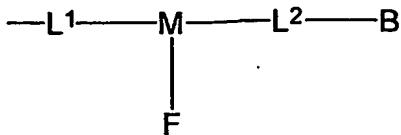
25 sulphur, –CH=CH–, >N–W wherein N is nitrogen and W is selected from hydrogen and the group R¹⁰;

A is selected from O and NR¹⁶ where R¹⁶ is the substituted amino radical:

30



at least one of groups R⁸, R⁹, R¹⁰, R¹¹, R¹², R¹³, R¹⁴, R¹⁵, R¹⁷ and R¹⁸ is the group:



- 5 where B, F, M, L¹ and L² are hereinbefore defined; remaining groups R¹¹, R¹², R¹³, R¹⁴ and R¹⁵ are independently selected from the group consisting of hydrogen, halogen, amide, cyano, nitro, amino, mono- or di-C₁ – C₆ alkyl-substituted amino, carbonyl, carboxyl, C₁ – C₆ alkyl, C₁ – C₆ alkoxy, aryl, heteroaryl, aralkyl and the group -(CH₂)_m-Y where Y is selected
- 10 from sulphonate, sulphate, phosphonate, phosphate and quaternary ammonium and m is zero or an integer from 1 to 6; remaining groups R⁸, R⁹ and R¹⁰ are independently C₁ – C₆ alkyl; remaining group R¹⁷ is selected from hydrogen, C₁ – C₄ alkyl and aryl; and remaining group R¹⁸ is selected from C₁ – C₆ alkyl, aryl, heteroaryl, an acyl
- 15 radical having from 2-7 carbon atoms, and a thiocarbamoyl radical.

Suitably, in the compounds according to formula (II) and (III), Z¹ and Z² may be selected independently from the group consisting of phenyl, pyridinyl, naphthyl, anthranyl, indenyl, fluorenyl, quinolinyl, indolyl, benzothiophenyl, benzofuranyl and benzimidazolyl moieties. Additional one, or two fused ring systems will be readily apparent to the skilled person. Preferably, Z¹ and Z² are selected from the group consisting of phenyl, pyridinyl, naphthyl, quinolinyl and indolyl moieties. Particularly preferred Z¹ and Z² are phenyl and naphthyl moieties.

25

Suitably, at least one of the groups R of the compounds of formula (II) and (III) is a water solubilising group for conferring a hydrophilic characteristic to the compound. Solubilising groups, for example, sulphonate, sulphonic acid and quaternary ammonium, may be attached directly to the aromatic ring structures Z¹ and/or Z² of the compounds of formula (II) and (III). Alternatively, solubilising groups may be attached by means of a C₁ to C₆ alkyl linker chain to said aromatic ring structures and may be selected from the group -(CH₂)_m-Y where Y is selected from sulphonate, sulphate,

phosphonate, phosphate, quaternary ammonium and carboxyl; and m is hereinbefore defined. Alternative solubilising groups may be carbohydrate residues, for example, monosaccharides, or polyethylene glycol derivatives.

Examples of water solubilising constituents include C₁ – C₆ alkyl sulphonates,

- 5 such as -(CH₂)₃-SO₃⁻ and -(CH₂)₄-SO₃⁻. However, one or more sulphonate or sulphonic acid groups attached directly to the aromatic ring structures of a dye of formula (II) or (III) are particularly preferred. Water solubility may be advantageous when labelling proteins.

- 10 In one embodiment the compound of formula (I) is a fluorescent reporter molecule. In this embodiment, none of the substituent groups R in the compounds of formula (II) and (III) contains a nitro group.

- In another embodiment, the compound of formula (I) is non-fluorescent
15 or substantially non-fluorescent dye wherein at least one of the groups R attached to the aromatic ring structures of the compounds of formula (II) and (III) comprises at least one nitro group. In this embodiment, suitably, the at least one nitro group may be attached directly to the Z¹ and/or Z² ring structures. In the alternative, a mono- or di-nitro-substituted benzyl group
20 may be attached to the Z¹ and/or Z² ring structures which optionally may be further substituted with one or more nitro groups. The non-fluorescent or substantially non-fluorescent cyanine dye or cyanine dye derivatives according to the invention may be used to label one component of a fluorescent donor/acceptor pair in assays involving the detection of binding
25 and/or cleavage events in reactions involving biological molecules, as described in EP 1086179 B1 (Amersham Biosciences UK Limited).

In the embodiments according to the first aspect:

- i) Aryl is an aromatic substituent containing one or two fused aromatic rings containing 6 to 10 carbon atoms, for example phenyl or naphthyl, the aryl being optionally and independently substituted by one or more substituents, for example halogen, straight or branched chain alkyl groups

containing 1 to 10 carbon atoms, aralkyl and alkoxy for example methoxy, ethoxy, propoxy and n-butoxy;

ii) Heteroaryl is a mono- or bicyclic 5 to 10 membered aromatic ring system containing at least one and no more than 3 heteroatoms which may be

5 selected from N, O, and S and is optionally and independently substituted by one or more substituents, for example halogen, straight or branched chain alkyl groups containing 1 to 10 carbon atoms, aralkyl and alkoxy for example methoxy, ethoxy, propoxy and n-butoxy;

iii) Aralkyl is a C₁ – C₆ alkyl group substituted by an aryl or heteroaryl

10 group;

iv) Halogen and halo groups are selected from fluorine, chlorine, bromine and iodine.

By virtue of the target bonding group F, the compounds according to

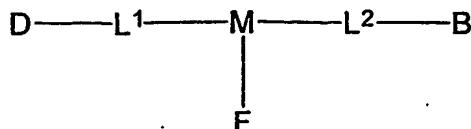
15 the present invention are useful for covalently labelling target biological materials in a site specific manner for applications in biological detection systems. Suitable target materials include proteins, post-translationally modified proteins, peptides, antibodies, antigens, and protein-nucleic acids (PNAs). The reporter moiety may also be conjugated to species which can

20 direct the path of the reporter within or aid entry to or exit from cells (live or dead); such as for example, long alkyl residues to allow permeation of lipophilic membranes, or intercalating species to localise a reporter in a nucleus or other cellular enclave containing double-stranded DNA.

25 In a second aspect, there is provided a method for labelling a protein of interest wherein said protein contains or is derivatised to contain an N-terminal cysteine, the method comprising:

i) adding to a liquid containing said protein a compound of formula (I):

30



(I)

wherein:

D is a dye selected from a cyanine dye or a derivative thereof;

B is an affinity tag;

F comprises a target bonding group selected from a carboxylic acid thioester
5 group and a 1,2-aminothiol group;

M is a group adapted for attaching to F; and

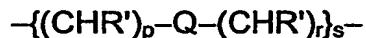
L¹ and L² each independently comprise a group containing from 1 – 40 linked
atoms selected from carbon atoms which may optionally include one or more
groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl

10 groups, where R' is selected from hydrogen and C₁ – C₄ alkyl; and

ii) incubating said compound with said protein under conditions suitable
for labelling said protein.

Suitably, there are 2 to 30 atoms in each of L¹ and L², preferably, 6 to
15 20 atoms.

Preferably, L¹ and L² are independently selected from the group:



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where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and
–CO–NH–; R' is hydrogen or C₁ – C₄ alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

Particularly preferred Q is selected from: –CHR'–, –O– and –CO–NH–,
25 where R' is hereinbefore defined.

Preferred compounds of formula (I) for use in labelling a target protein
are those having formula (II) or (III) as hereinbefore defined.

30 Covalent labelling using compounds of the present invention may be
accomplished with a target having at least one carboxylic acid thioester group
or 1,2-aminothiol group as hereinbefore defined. The target may be incubated

with an amount of a compound of the present invention having at least one group F as hereinbefore defined that can covalently bind with the complementary group of the target material. The target material and the compound of the present invention are incubated under conditions and for a 5 period of time sufficient to permit the target material to covalently bond to the compound of the present invention. Thus, for example, the thioester group F may be reacted and form a covalent bond with any of the above target materials that contains, or has been derivatised to contain, a 1,2-amino thiol group. These methods and the products resulting from them, for example, 10 reporter-labelled biomolecules are envisaged as further aspects of the invention.

Suitably, the protein of interest may be selected from the group consisting of antibody, antigen, protein, peptide, microbial materials, cells and 15 cell membranes.

In a particular embodiment according to the second aspect, there is provided a method of separating and/or purifying the dye-labelled protein of interest by affinity chromatography utilising the affinity of the affinity tag moiety 20 for an immobilised ligand (or specific binding partner) attached to a support material. Affinity chromatography provides a quick and convenient method to enable the separation of labelled and unlabelled protein molecules under physiological conditions. Proteins labelled with an affinity tag can be selectively bound to an affinity column and any unreacted protein removed by 25 washing the column. Suitable specific binding moieties include avidin or streptavidin (for a biotin tag); immobilised metal ions, for example, Cu(II), Ni(II), Fe(II) and Fe(III) (for His-tag or iminodiacetic acid). Methods for affinity purification of proteins will be well known to the skilled person, see for example Ostrove, S, Methods in Enzymology, (1990), Vol 182, page 357.

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In a typical labelling procedure, a target peptide or protein containing an N-terminal cysteine residue is agitated with an excess of a cyanine dye thioester derivative, e.g. Cy5-MESNA (Cy5-mercaptoethanesulphonic acid

ester), in phosphate buffer (typically 200 mM NaCl, 200 mM sodium phosphate) at ~pH 7.3 – 7.4 containing ~1.5% MESNA. The concentration of the target polypeptide in the labelling reaction is generally between 100 µM to 10 mM, whilst the Cy5-MESNA is generally present in excess, for example 1.5
5 to 3-fold molar excess. When the target polypeptide concentration is relatively low the concentration of Cy5-MESNA is usually maintained at or above 1 mM. Generally, for labelling small peptides a solution of Cy5-MESNA and MESNA cofactor is directly added to the lyophilised target.

10 Typically, for site specific labelling of proteins and large polypeptides using the reagents of the present invention, the target is first exchanged into an appropriate buffer, which is known not to affect the labelling reaction. An equal volume of a solution of Cy5-MESNA and MESNA thiol cofactor in ligation buffer is then added to the protein to give the desired final
15 concentration of the reactants. The reaction mixture is agitated overnight at room temperature. The reaction time may be lowered to less than one hour for high reactant concentrations or, if the stability of the target polypeptide is an issue, the labelling reaction may be performed efficiently at 4°C. On completion of the labelling reaction, dithiothreitol (DTT) is added to a final
20 concentration of ~50 mM and the desired material isolated by affinity chromatography.

Various different denaturants, organic solvents and detergents may be added to the reaction buffer when performing native chemical ligation and
25 expressed protein ligation reactions, to aid the ligation of the peptide fragments and/or stabilise the reactants or products. Such reagents may be utilised in the labelling reaction to increase product yield if necessary. Examples include, but are not limited to guanidinium chloride, urea, dimethylformamide, dimethylsulphoxide, acetonitrile, triton X-100, octyl
30 glucoside, 1,6-hexanediol and glycerol.

The ligation reaction using the derivatised cyanine dye according to the present invention may be optimally performed at between pH 7.0 and pH 8.0

and at temperatures varying between 4°C and 37°C. It is envisaged that such a range of conditions are compatible to the site-specific labelling reaction described herein.

5 The advantage of the present method is that it enables the introduction of an extrinsic label into a proteinaceous substrate in a regioselective and specific manner, thus minimising any detrimental effects that labelling may have on the biological function of the protein. The importance of controlling stoichiometry of labelling is important where dye overload may interfere with
10 biological activity. In addition, if this controlled labelling stoichiometry is directed towards a single terminal site, rather than towards an internal site, this may have the benefit of further maintaining the biological viability of the labelled species.

15 The invention is further illustrated by reference to the following examples and figure in which:

Figure 1 illustrates the products from the labelling reaction of an N-terminal cysteine derivative of the Grb2SH2 domain with the thioester derivative, α-D-
20 desthiobiotin-ε-Cy5-L-lysine-MESNA according to Examples 3 and 4.

Experimental

1. 2-[*(1E,3E,5E)-5-(3,3-Dimethyl-1-{6-oxo-6-[*(2-sulphoethyl)thio]hexyl}-5-**
25 sulfo-1,3-dihydro-2*H*-indol-2-ylidene)penta-1,3-dienyl]-1-ethyl-3,3-dimethyl-5-
sulfo-3*H*-indolium



To CyTM5 mono acid (47mg, 0.062mmol) in a solution of 7-azobenzotriazolyoxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP, 66mg, 0.127mmol) in anhydrous dimethylformamide (DMF, 1ml) was added anhydrous di-isopropylethylamine (DIEA)(30µl, 0.1724mmol) and mixed for 5 minutes. The activated dye solution was then added to a stirred solution of 2-mercaptoethanesulphonic acid, sodium salt (MESNA, 40mg, 0.243mmol) in DMF (2mls) and DIEA (30µl, 0.1724mmol) under a dry nitrogen atmosphere. To this mixture was added as a solid, dried 4A molecular sieves(~1g, <5micron, activated powder). The mixture was stirred under a dry nitrogen atmosphere, at room temperature, in the dark overnight. Thin layer chromatography analysis (reverse phase C18 plates, eluents water/acetonitrile (70:30, containing 0.1% TFA) indicated a major component, R_f _{thioester} = 0.25) with no trace of starting material (R_f _{acid} = 0.12).

The molecular sieves were removed by filtration and filtrate was added dropwise into an excess of ethyl acetate, the blue solid was filtered off and was purified by reverse phase-high performance liquid chromatography (RP-HPLC); [Phenomenex Prodigy C18 column; 15%B-30%B over 30 mins @ 20 ml/min; eluent A = 0.1%TFA/water, eluent B = 0.1%TFA.MeCN, UV detection at 650nm]. The product was isolated as a dark blue/purple solid (40 mg, 0.0513mmol, 83 % yield).

Accurate mono-isotopic mass: C₃₅H₄₅O₁₀N₂S₄ requires 781. Found Maldi Tof, LC-MS found mass: M+ 781.25. δ H (300MHz, d6-DMSO): 8.37 (t, 1H), 8.36 (t, 1H), 7.83 (d, 1H), 7.82 (d, 1H), 7.67(dd, 1H), 7.64 (dd, 1H), 7.36 (d, 1H), 7.33 (d, 1H), 6.61 (t, 1H), 6.38 (d, 1H), 6.28 (d, 1H), 4.15 (m, 2H), 4.08 (t, 2H), 3.06 (m, 2H), 2.63 (m, 2H), 2.56 (t, 2H), 1.64 (m, 2H), 1.28(t, 3H, 7.1), 1.40 (m, 2H). λ_{max} (abs) = 647nm. (ε (H₂O) = 230,000M⁻¹cm⁻¹).

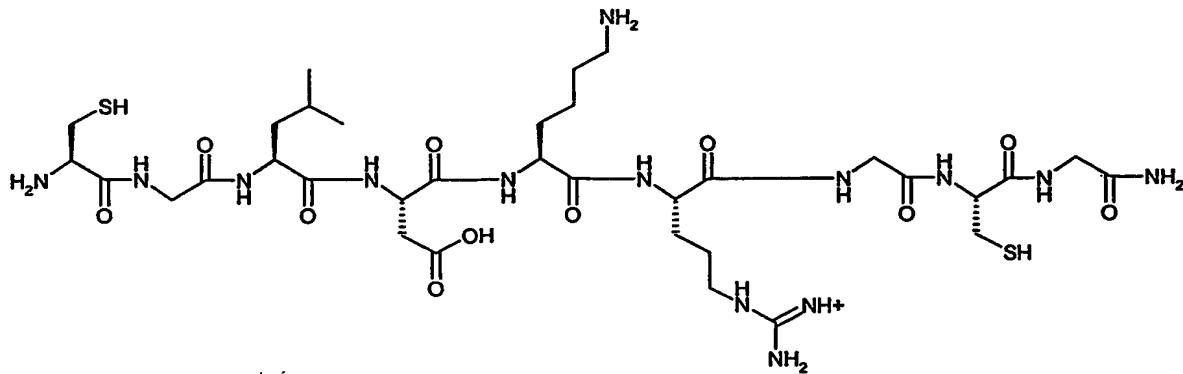
2. Determination of Specificity of Labelling using 2-[*(1E,3E,5E)-5-(3,3-dimethyl-1-[6-oxo-6-[*(2-sulphoethyl)thio]hexyl]-5-sulfo-1,3-dihydro-2H-indol-2-ylidene)penta-1,3-dienyl]-1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium**

5 2.1 Preparation of Cy5-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-NH₂

i) Synthesis of H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Lys(Boc)-Arg(Pmc)-Gly-Cys(Trt)-Gly-rink amide resin

10 H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Lys(Boc)-Arg(Pmc)-Gly-Cys(Trt)-Gly-rink amide resin was synthesised using a commercially available Applied Biosystems Model 433A automated peptide synthesiser using FastMoc™ chemistry, following the instrument manufacturer's recommended procedures throughout. The peptide was synthesised on a 0.25 millimolar scale
15 employing O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the activating agent.

ii) H-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH₂



20 H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Lys(Boc)-Arg(Pmc)-Gly-Cys(Trt)-Gly-rink amide resin (100mg, theoretical loading 0.36mmol/g) was deprotected and cleaved from solid phase in 95% trifluoroacetic acid (TFA) / 2.5% tri-isopropylsilane (TIS) / 2.5% water (3 mls) at room temperature for 2 hours. The crude product was precipitated into a 10 fold excess of cold diethyl ether, 25 centrifuged at 2500 rpm for 5 minutes and the ether decanted off. The crude

peptide was washed twice more with ether and was purified by reverse phase-high performance liquid chromatography (RP-HPLC) [Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient : 0-73%B over 30 mins @1ml/min, detection at 214nm]. The product
5 was isolated and lyophilised to afford a colourless fluffy solid (21mg by weight, 60%). Mono-isotopic mass: 906.4. Found mass (LC-MS): MH+ @ 907.3; M+Na @ 929.6; > 95% pure as judged by RP-HPLC @ 214nm (Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, 5-50% B over 25mins @ 1ml /min, UV detection at 650nm).

10

iii) Cy5-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH₂

To solid H-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH₂ (3.0mg by weight, 0.0033mmol) was added a solution of Cy5-MESNA (3.5mg,
15 0.0045mmol) in 200mM phosphate buffer, 200mM NaCl pH 7.2 containing 1.5% 2-mercaptopethanesulphonic acid, sodium salt (400μl). The reaction mixture was stirred on rollers for 30 minutes at room temperature in darkness. During incubation, a blue precipitate formed, which re-dissolved on addition of acetonitrile (40μl).

20

500mM DTT (200μl) in 200mM phosphate buffer; 200mM NaCl pH 7.2 (0.5mls, 0.0025mmol) was then added to the reaction mixture, with complete mixing and was stirred for a further 30 minutes at room temperature in the dark. The crude reaction mixture was then purified by RP-HPLC
25 [Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 20-35%B over 30 mins at 4 ml/min, detection at 650nm and 214nm]. The product was isolated and lyophilised as a blue fluffy solid (1.6 mg by UV/VIS at 650nm; 50% Yield; 98% pure as judged by RP-HPLC at 650nm. Mono-isotopic mass C₆₇H₁₀₁N₁₆O₁₈S₄ requires
30 1545.636. Found (LC-MS) M+ 1545.7.

2.2 Characterisation of Labelled Peptide

i) Ellman's Test on Cy5-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH₂

5 A sample of Cy5-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH₂ was dissolved in 100mM sodium phosphate buffer; 1mM EDTA pH 7.27 (stock buffer) to afford a 0.3μM peptide stock by UV/VIS at 650nm.

10 0.3μM peptide stock (40μl) and 10mM 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) in 100mM sodium phosphate buffer; 1mM EDTA pH 7.27 (50μl) were mixed together in stock buffer (910μl) to afford a green solution. The absorbance at 412nm (due to generation of TNB²⁻) was recorded against a DTNB blank [10mM DTNB stock (50μl) in stock buffer (950μl)]. Using the known molar absorption coefficient of TNB²⁻ (14150M⁻¹cm⁻¹), the thiol concentration was determined as 655μM, approximately twice the peptide concentration, confirming two free thiol groups. [SH] = [A412nm (sample)-A412nm(reference)/ε (TNB²⁻)

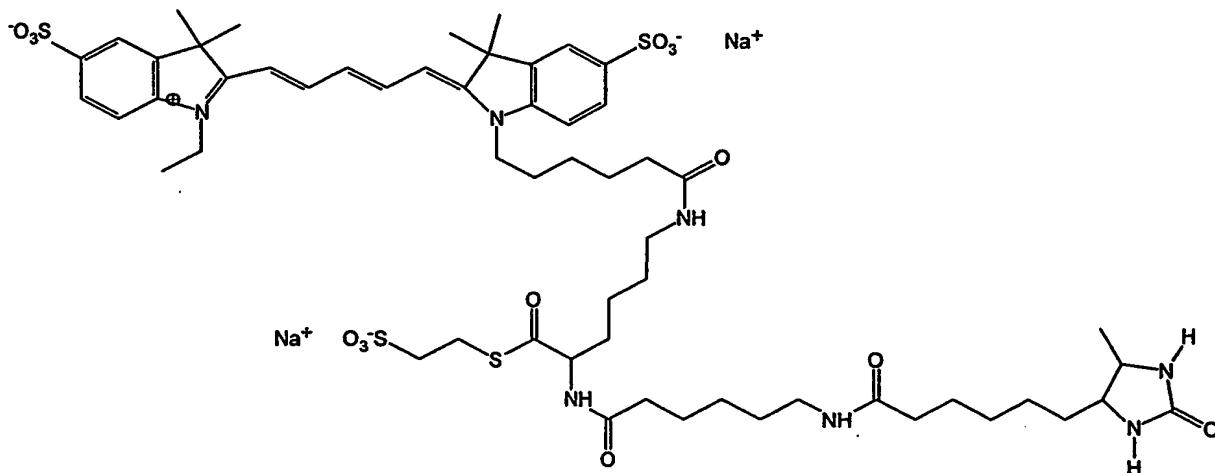
ii) Enzyme Digestion of Cy5-Cys-Gly-Leu-Asp-Lys-Arg -Gly-Cys-Gly-NH₂

20 To a solution of Cy5-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH₂ (180μg by UV/VIS at 650nm) in TRIS buffer pH 8.0 (100μl) containing 10% acetonitrile was added Asp-N (2μg) in TRIS buffer pH 8.0 (70μl). The reaction mixture was stirred at room temperature in the dark for 4 hours. The reaction 25 mixture was treated with 250mM Tris (2-carboxyethyl)phosphine, HCL (TCEP) in TRIS buffer pH 8.0 (55μl) for 30 minutes. The reduced reaction mixture was then diluted 1:5 with 0.1%TFA in water and purified by reverse phase HPLC [Phenomenex Jupiter C18, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, 5-50% B over 30mins @ 1ml /min, UV at 214nm, 30 650nm]. The two components of the reaction mixture were identified as : Cy5-Cys-Gly-Leu-OH, mono-isotopic mass: C₄₄H₆₀N₅O₁₁S₃ requires 930.3451. Found mass (MALDI Tof): M+ 930.0 and H-Asp- Lys-Arg-Gly-Cys-Gly-NH₂,

monoisotopic mass: C₂₃H₄₃N₁₁O₈S requires 633.3016, found mass (MALDI-Tof): M+ 633.0.

3. Preparation of α-D-Desthiobiotin-ε-Cy5-L-lysine-MESNA [N⁶-(6-{(2Z)-2-

[(2E,4E)-5-(1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium-2-yl)penta-2,4-dienylidene]-3,3-dimethyl-5-sulfo-2,3-dihydro-1H-indol-1-yl}hexanoyl)-N²-(6-{[6-(5-methyl-2-oxoimidazolidin-4-yl)hexanoyl]amino}hexanoyl) lysylthioethane-2-sulfonic acid]



20 3.1 Preparation of α-Fmoc-ε-Cy5-L-lysine-OH [2-[(1E,3E)-5-(1-[6-[(5-carboxy-5-[(9H-fluoren-9-ylmethoxy)carbonyl]amino]pentyl)amino]-6-oxohexyl)-3,3-dimethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene)-1,3-pentadienyl]-1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium salt]

25 Cy5 mono free acid potassium salt (Amersham Pharmacia Biotech Ltd) (450mg, 0.65 mmol) and DIEA (720μl) were dissolved in anhydrous dimethylsulphoxide (18ml). To this was added O-(N-succinimidyl)-N,N,N',N'-bis(tetramethylene)-uronium hexafluorophosphate (666mg, 1.6mmol) and the reaction mixture stirred at room temperature for 1 hr after which time
30 negligible starting material remained by TLC (RPC₁₈, 1:1 methanol:water). The reaction mixture was slowly poured into diethyl ether to precipitate the product; Cy5 mono NHS ester, which was filtered off, washed with ethyl acetate and dried *in vacuo*. The product was re-dissolved in anhydrous

dimethylsulphoxide (18ml) and DIEA (720 μ l) added. Fmoc-lysine-OH (360mg, 0.98mmol) was suspended in a mixture of phosphate buffer (pH 7.4) (9ml) and dimethylsulphoxide (9ml). The suspension was slowly added to the solution of Cy5 NHS ester. The reaction mixture was stirred at room
5 temperature for 12 hours. TLC (RPC₁₈, 2:3 methanol:water) showed the disappearance of starting material and the formation of a new product spot.

The product was purified by HPLC (Dynamax C18 column (50 x 4.14cm); flow rate 25ml/min; gradient of 20 to 80% B over 80 mins (eluent A = 10 0.1% TFA in water and eluent B = 0.1% TFA in acetonitrile); detection at 650nm. The fractions containing the desired product were pooled and most of the solvent removed under reduced pressure, the residue was freeze dried. The product; α -Fmoc- ϵ -Cy5-L-lysine-OH was obtained as a fluffy cyan solid (487mg, 74%). MS (MALDI TOF) found 1008(M $^+$); [theoretical 15 (C₅₄H₆₄N₄O₁₁S₂) 1009]. ¹H NMR (200 MHz D₆DMSO) 1.27(t, 3H, CH₃), 1.35 (m, 4H, CH₂, CH₂), 1.55 (m's, 4H, CH₂, CH₂), 1.7 (s, 12H, (CH₃)₂), 1.78 (m, 2H, CH₂), 2.05 (t, 2H, CH₂), 3.0, (m, 2H, CH₂), 3.92 (m, 1H CH amino acid), 4.11 (m, 4H, N-CH₂, N $^+$ CH₂), 4.27 (m 3H O-CH₂, CH fluorenyl), 6.3 (d, 2H, α , α' methine), 6.59, (t, 1H, γ methine) 7.28-7.48 (m's, 6H, Fmoc and indole Ar), 20 7.65 (d, 2H, fluorenyl Ar), 7.73 (d, 2H, fluorenyl Ar), 7.85 (s, 2H, indole Ar), 7.9 (d, 2H, indole Ar), 8.38 (t, 2H, β , β' methine).

3.2 Preparation of ϵ -Cy5-L-lysine-OH [N⁶-(6-((2E)-2-[(2E,4E)-5-(1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium-2-yl)penta-2,4-dienylidene]-3,3-dimethyl-5-sulfo-2,3-dihydro-1H-indol-1-yl]hexanoyl)lysine]

α -Fmoc- ϵ -Cy5-L-lysine-OH (100mg, 0.1mmol) was deprotected in a mixture of 20% piperidine in NMP (2ml). TLC (RP C18; 1:1 MeOH:water) showed the formation of a new product spot, rf = 0.92 as compared to that of 30 the starting material, rf = 0.46. The piperidine was removed under reduced pressure and the dye precipitated by pouring the reaction mixture into diethyl ether. The product was filtered off and washed with dichloromethane and

then ethyl acetate to remove the yellow Fmoc derived by-product. The product was dissolved in water, filtered and then purified by HPLC; [Vydac Protein and Peptide C18 column; 0-50%B over 45mins at 10ml/min; eluent A = 0.1% TFA/water, eluent B = 0.1% TFA/MeCN, detection at 215nm).

- 5 Fractions containing the desired product were combined and the solvents removed under reduced pressure to leave a blue residue. The residue was triturated with ethyl acetate and the resultant solid dried under vacuum at 40°C. The product; ϵ -Cy5-L-lysine-OH was obtained as a dark blue solid (43mg, 48%). Analytical HPLC AKTA analysis; Phenomenex C18 column; 0-
10 50%B over 30mins at 1ml/min; eluent A = 0.1% TFA/water, eluent B = 0.1% TFA/MeCN, detection at 650nm; rt = 20.22mins. MS (MALDI TOF) found 785 (M⁺); [theoretical (C₃₉H₅₃N₄O₉S₂) 785]. ¹H NMR (300 MHz D₆DMSO) 1.26 (t, 3H, CH₃), 1.52 (m, 4H, CH₂, CH₂), 1.62 (m, 4H, CH₂, CH₂), 1.69 (s, 12H, (CH₃)₂), 2.02 (m, 2H, CH₂), 2.92 (m, 2H, CH₂), 3.85 (m, 1H, CH amino acid),
15 4.10 (m, NCH₂, N⁺CH₂), 6.29 (d, 1H α methine), 6.34 (d, 1H, α' methine), 6.58 (t, 1H, γ methine), 7.32 (m, 2H, indole Ar), 7.82 (d, 2H, indole Ar), 8.04 (m, 3H, NH₃⁺), 8.37 (t, 2H, β , β' methine).

3.3 Preparation of D-Desthiobiotinamidocaproic acid

- 20 D-Desthiobiotin (250mg, 1.17mmol) was dissolved in anhydrous dimethylsulphoxide (2ml). To this solution was added PyAOP (610mg, 1.17mmol) and DIEA (200 μ l, 1.15mmol). The mixture was stirred under nitrogen at RT for 3hrs before adding 6-aminocaproic acid (153mg, 1.17mmol)
25 and a further amount of DIEA (200 μ l, 1.15mmol). The reaction mixture was stirred for a further 4hrs. TLC (RP C18; 1:2 MeOH:water; detection by cinnamaldehyde staining) showed the formation of a new product spot, rf = 0.63 as compared to the starting material, rf = 0.76). The reaction mixture was poured into excess diethyl ether to give a brown oil. The oil was triturated
30 with ethyl acetate until an off-white solid was obtained. The product was filtered off and purified by HPLC [Vydac Protein and Peptide C18 column; 0-50%B over 30mins at 10ml/min; eluent A = 0.1% TFA/water, eluent B = 0.1% TFA/MeCN, detection at 215nm). Fractions containing the desired product

were pooled and the solvents removed under reduced pressure. The residue was triturated with ethyl acetate to give a white solid. The product was filtered off and dried under reduced pressure at 40°C. The product; D-desthiobiotinamidocaproic acid, was obtained as a white solid (48mg, 12.5%).

5 MS (MALDI TOF) found 327(M⁺); [theoretical (C₁₆H₂₉N₃O₄) 327]. ¹H NMR (300 MHz D₆DMSO) 0.96 (d, 3H, CH₃), 1.25 (m, 6H, CH₂, CH₂, CH₂), 1.34 (m, 4H, CH₂, CH₂), 1.48 (m, 4H, CH₂, CH₂), 2.03 (m, 2H, C(O)CH₂), 2.60 (m, 2H, C(O)CH₂), 3.01 (m, 2H, NHCH₂), 3.47 (m, 1H, CH), 3.60 (m, 1H, CH), 6.11 (s, 1H, NH), 6.29 (s, 1H, NH), 7.71 (s, 1H, NH).

10

3.4 Preparation of D-Desthiobiotinamidocaproate N-hydroxy succinimidyl ester

D-Desthiobiotinamidocaproic acid (48mg, 0.147mmol) was dissolved in
15 DMF (1ml) and N,N,N',N'-bis(tetramethylene)-O-(N-succinimidyl)uronium hexafluorophosphate (HSPyU) (90mg, 0.21mmol) and DIEA (40μl, 0.23mmol) were added. The reaction mixture was stirred under nitrogen at RT for 6hrs, TLC (RP C18; 1:2 MeOH:water; materials detected by cinnamaldehyde staining) showed the formation of a new product at the base line as compared
20 to the starting material, rf = 0.68. The reaction mixture was poured into diethyl ether to give a brown gum. The supernatant was decanted off and the gum again treated with diethyl ether. No solid formed. The gum was dried under reduced pressure and the product, D-desthiobiotinamidocaproate N-hydroxy succinimidyl ester was used directly in the next dye coupling reaction,
25 assuming a theoretical yield of 62mg.

3.5 Preparation of α-D-Desthiobiotin-ε-Cy5-L-lysine-OH [N⁶-(6-{(2Z)-2-[
30 (2E,4E)-5-(1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium-2-yl)penta-2,4-
dienylidene]-3,3-dimethyl-5-sulfo-2,3-dihydro-1H-indol-1-yl}hexanoyl)-N²-(6-
{[6-(5-methyl-2-oxoimidazolidin-4-yl)hexanoyl]amino}hexanoyl)lysine]

ε-Cy5-L-lysine-OH (43mg, 0.048mmol), D-desthiobiotinamidocaproate N-hydroxy succinimidyl ester (62mg, 0.146mmol) and DIEA (80μl, 0.45mmol).

were stirred together in DMF (2ml) for 3hrs. TLC (RP C18; 1:1 MeOH:water) showed the formation of a new product spot, $rf = 0.79$, just under that of the starting material. The product was precipitated into diethyl ether (200ml) and then filtered off. The material was purified in multiple runs by HPLC [Vydac

- 5 Protein and Peptide C18 column; eluent A = 0.1% TFA/water, eluent B = 0.1% TFA/MeCN, various gradients, detection at 215nm) until the material was seen to be pure by 1H NMR. Analytical HPLC AKTA analysis; Phenomenex C18 column; 0-50%B over 30mins at 1ml/min; eluent A = 0.1% TFA/water, eluent B = 0.1% TFA/MeCN, detection at 650nm; rt = 22.04mins. MS (MALDI
10 TOF) found 1094 (M^+); [theoretical ($C_{55}H_{80}N_7O_{12}S_2$) 1094].

3.6 Preparation of α -D-Desthiobiotin- ϵ -Cy5-L-lysine-MESNA [N^6 -(6-{(2Z)-2-[(2E,4E)-5-(1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium-2-yl)penta-2,4-dienylidene]-3,3-dimethyl-5-sulfo-2,3-dihydro-1H-indol-1-yl}hexanoyl)- N^2 -(6-{[6-(5-methyl-2-oxoimidazolidin-4-yl)hexanoyl]amino}hexanoyl) lysylthio propane-3-sulfonic acid]

α -D-Desthiobiotin- ϵ -Cy5-L-lysine-OH (10mg, 8.8 μ mol) was dissolved in anhydrous dimethylsulphoxide (2ml), PyAOP (10mg, 19.2 μ mol), MESNA

- 20 (5mg, 0.30mmol) and DIEA (10 μ l, 0.06mmol) were added and the reaction mixture was stirred under nitrogen for 4hrs. The reaction mixture was purified by RP-HPLC; [Vydac Protein and Peptide C18 column; 15-40%B over 45mins at 10ml/min; eluent A = 0.1% TFA/water, eluent B = 0.1% TFA/MeCN, detection at 215nm). The product containing fractions were combined and the 25 majority of solvent removed under reduced pressure, the residue was freeze dried. The product was obtained as a fluffy blue solid (4mg, 37%). TLC; RP C18; 1:1 water:acetonitrile) $rf = 0.76$. Analytical HPLC AKTA analysis; Phenomenex C18 column; 0-50%B over 30mins at 1ml/min; eluent A = 0.1% TFA/water, eluent B = 0.1% TFA/MeCN, detection at 650nm; rt = 21.04mins.
30 $\lambda_{max} = 648nm$ (PBS buffer). MS (MALDI TOF) found 1219 (MH^+); [theoretical ($C_{57}H_{84}N_7O_{14}S_4$) 1218].

4. Protein labelling and affinity purification

4.1 Labelling of N terminal cysteine Grb2SH2 with α -D-desthiobiotin- ϵ -Cy5-L-lysine-MESNA and purification

5

To N-terminal cysteine Grb2SH2 (N-Cys-Grb2SH2) (200 μ M in PBS buffer pH 7.2) (200 μ l) was added α -D-desthiobiotin- ϵ -Cy5-L-lysine-MESNA (2mM in reaction buffer) (200 μ l). N-Cys-Grb2SH2 was prepared using recombinant techniques. The reaction buffer consisted of phosphate buffer (200mM), pH 7.2 containing sodium chloride (200mM) and 4% MESNA. The reaction mixture was left at RT for 12 hrs, wrapped in foil to protect from light. The reaction was then quenched with dl-dithiothreitol (final concentration 60mM). Unreacted dye was separated from labelled/unlabelled protein by FPLC, using a fast desalt column and eluent of PBS buffer, pH 7.4; 2ml/min, detection 280 nm. Protein fractions were combined and desthiobiotin- ϵ -Cy5-L-lysine affinity probe labelled protein was bound to streptavidin beads (PIERCE UltralinkTM streptavidin). The beads were washed vigorously with both PBS buffer and binding buffer (PBS containing 500mM NaCl). The product; α -D-desthiobiotin- ϵ -Cy5-L-Lys-Cys-Grb2SH2 was extracted from the streptavidin beads by adding cold biotin (1.6mM). Several extraction runs were required. The materials were further purified by dialysis (PIERCE Slide-a-lyserTM mini dialysis units, 7,000 mwco) to remove free biotin from the sample. The product was analysed by SDS PAGE together with the following controls (see Figure1):

25

30

- | | |
|-------------|--|
| Lane 1 | MW marker |
| Lane 2 | Unligated protein control |
| Lane 3 | Ligation reaction mixture: α -D-desthiobiotin- ϵ -Cy5-L-Lysine-MESNA and N-Cys-Grb2SH2 |
| Lane 4 | Labelled/unlabelled N-Cys-Grb2SH2 after FPLC purification |
| Lane 5 | Unbound protein |
| Lane 6 | Streptavidin bead washes |
| Lanes 7,8,9 | Affinity purified product |
| Lane 10 | Unreacted α -D-desthiobiotin- ϵ -Cy5-L-Lysine-MESNA |

The gel was imaged using a Typhoon imager (Figure 1B) using parameters for Cy5 fluorescence to detect fractions containing the fluorescent label. The gel was then stained with Coomassie blue stain (Figure 1A) to determine the protein containing fractions. SDS PAGE gel shows that (a) unlabelled protein (both factor XA and N-Cys-Grb2SH2 did not bind to the streptavidin beads (Figure 1A and 1B, column 5) (enriched protein stain) and (b) the product was removed from the streptavidin beads by adding cold biotin (Figure 1A and 1B, columns 7 and 8) (both protein stain and Cy5 fluorescence).

10

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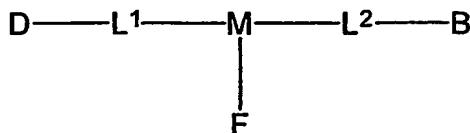
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Claims

1. A compound comprising a cyanine dye or derivative thereof containing at least one target bonding group selected from a carboxylic acid thioester group or a group suitable for covalent reaction with a thioester, characterised in that said compound includes an affinity tag covalently bound thereto.

5 2. A compound according to claim 1 having the formula (I):

10



(I)

15 wherein:

D is a dye selected from a cyanine dye or a derivative thereof;

B is an affinity tag;

F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group;

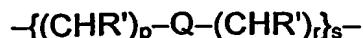
20 M is a group adapted for attaching to F; and

L¹ and L² each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C₁ – C₄ alkyl.

25

3. A compound according to claim 2 wherein each of L¹ and L² contains from 2 to 30 atoms.

4. A compound according to claim 2 wherein L¹ and L² are independently selected from the group:



where Q is selected from: $-\text{CHR}'-$, $-\text{NR}'-$, $-\text{O}-$, $-\text{CH}=\text{CH}-$, $-\text{Ar}-$ and $-\text{CO}-\text{NH}-$; R' is hydrogen or $\text{C}_1 - \text{C}_4$ alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

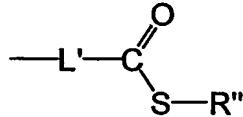
5. A compound according to claim 4 wherein Q is selected from $-\text{CHR}'-$,
5 –O– and $-\text{CO}-\text{NH}-$, where R' is hereinbefore defined.

6. A compound according to any of claims 1 to 5 wherein said affinity tag
is selected from biotin and desthiobiotin.

10 7. A compound according to any of claims 1 to 5 wherein said affinity tag
is selected from his-tag, iminodiacetic acid and nitrilotriacetic acid.

8. A compound according to any of claims 1 to 7 wherein the target
bonding group F is a carboxylic acid thioester of formula:

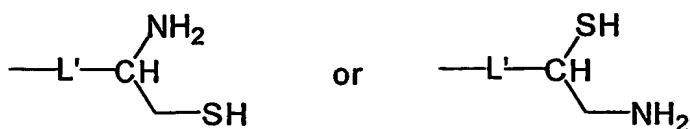
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wherein L' is a bond or is a group containing from 1 – 30 linked atoms
20 selected from carbon atoms and optionally one or more groups selected from
 $-\text{NH}-$, $-\text{O}-$ and $-\text{CO}-\text{NH}-$; and R'' is $\text{C}_1 - \text{C}_4$ alkyl, $\text{C}_6 - \text{C}_{10}$ aryl, or $\text{C}_7 - \text{C}_{15}$
aralkyl, which may be optionally substituted with sulphonate; or is the group
 $-(\text{CH}_2)_2-\text{CONH}_2$.

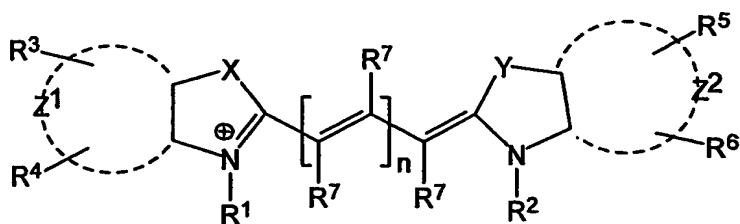
25 9. A compound according to any of claims 1 to 7 wherein the target
bonding group F is a 1,2-aminothiol group of formula:

30



wherein L' is hereinbefore defined.

10. A compound according to any of claims 1 to 9 wherein the compound has the formula (II):



10 (II)

wherein:

groups R^3 and R^4 are attached to the Z^1 ring structure and groups R^5 and R^6 are attached to the Z^2 ring structure;

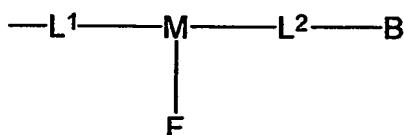
n is an integer from 1 to 3;

15 Z^1 and Z^2 independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

X and Y are the same or different and are selected from: $>CR^8R^9$, oxygen,

20 sulphur, $-CH=CH-$, $>N-W$ wherein N is nitrogen and W is selected from hydrogen and the group R^{10} ;

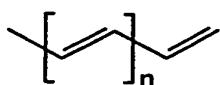
at least one of groups R^1 , R^2 , R^3 , R^4 , R^5 , R^6 , R^8 , R^9 and R^{10} is the group:



where B , F , M , L^1 and L^2 are hereinbefore defined;

groups R^7 are independently selected from hydrogen and $C_1 - C_4$ alkyl which may be unsubstituted or substituted with aryl, or two or more of R^7 together

30 with the group:



form a hydrocarbon ring system substituted with R⁷ and which may optionally contain a heteroatom selected from -O-, -S- or >NR⁷, wherein R⁷ and n are hereinbefore defined;

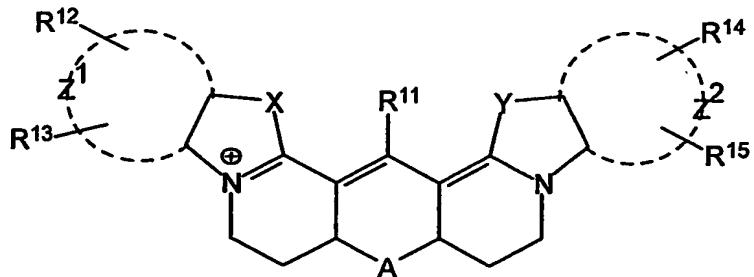
remaining groups R³, R⁴, R⁵ and R⁶ are independently selected from the

5 group consisting of hydrogen, halogen, amide, cyano, nitro, mono- or di-C₁ – C₆ alkyl-substituted amino, carbonyl, carboxyl, C₁ – C₆ alkyl, C₁ – C₆ alkoxy, aryl, heteroaryl, aralkyl and the group -(CH₂)_m-Y where Y is selected from sulphonate, sulphate, phosphonate, phosphate and quaternary ammonium and m is zero or an integer from 1 to 6;

10 remaining groups R⁸, R⁹ and R¹⁰ are independently C₁ – C₆ alkyl; and remaining groups R¹ and R² are independently selected from hydrogen, C₁ – C₁₀ alkyl, the group -(CH₂)_m-Y wherein Y and m are hereinbefore defined, and benzyl which may be unsubstituted or substituted by up to two nitro groups.

15

11. A compound according to any of claims 1 to 9 wherein the compound has the formula (III):



20

(III)

wherein

groups R¹², R¹³, R¹⁴ and R¹⁵ are attached to the rings containing X and Y or, optionally are attached to atoms of the Z¹ and Z² ring structures;

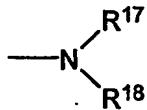
Z¹ and Z² independently represent the atoms necessary to complete one ring

30 or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

X and Y are the same or different and are selected from: >CR⁸R⁹, oxygen, sulphur, -CH=CH-, >N-W wherein N is nitrogen and W is selected from hydrogen and the group R¹⁰;

A is selected from O and NR¹⁶ where R¹⁶ is the substituted amino radical:

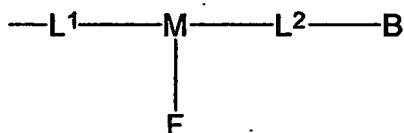
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at least one of groups R⁸, R⁹, R¹⁰, R¹¹, R¹², R¹³, R¹⁴, R¹⁵, R¹⁷ and R¹⁸ is the

10

group:



15

where B, F, M, L¹ and L² are hereinbefore defined; remaining groups R¹¹, R¹², R¹³, R¹⁴ and R¹⁵ are independently selected from the group consisting of hydrogen, halogen, amide, cyano, nitro, mono- or di-C₁ – C₆ alkyl-substituted amino, carbonyl, carboxyl, C₁ – C₆ alkyl, C₁ – C₆ alkoxy, aryl, heteroaryl, aralkyl and the group -(CH₂)_m-Y where Y is selected

20

from sulphonate, sulphate, phosphonate, phosphate and quaternary ammonium and m is zero or an integer from 1 to 6;

remaining groups R⁸, R⁹ and R¹⁰ are independently C₁ – C₆ alkyl;

remaining group R¹⁷ is selected from hydrogen, C₁ – C₄ alkyl and aryl; and

remaining group R¹⁸ is selected from C₁ – C₆ alkyl, aryl, heteroaryl, an acyl

25

radical having from 2-7 carbon atoms, and a thiocarbamoyl radical.

12. A compound according to claim 10 or claim 11 wherein Z¹ and Z² are selected independently from the group consisting of phenyl, pyridinyl, naphthyl, quinolinyl and indolyl moieties.

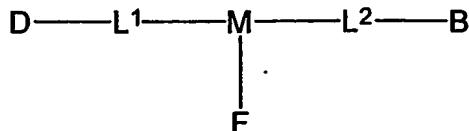
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13. A compound according to claim 10 or claim 11 wherein Z¹ and Z² are selected from phenyl and naphthyl moieties.

14. A method for labelling a protein of interest wherein said protein contains or is derivatised to contain an N-terminal cysteine, the method comprising:

- i) adding to a liquid containing said protein a compound of formula (I):

5



(I)

10 wherein:

D is a dye selected from a cyanine dye or a derivative thereof;

B is a bioaffinity tag;

F comprises a target bonding group selected from a carboxylic acid thioester group and a 1,2-aminothiol group;

15 M is a group adapted for attaching to F; and

L¹ and L² each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C₁ – C₄ alkyl; and

20 ii) incubating said compound with said protein under conditions suitable for labelling said protein.

15. A compound according to claim 14 wherein each of L¹ and L² contains from 2 to 30 atoms.

25

16. A method according to claim 14 wherein L¹ and L² are independently selected from the group:



30

where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and –CO–NH–; R' is hydrogen or C₁ – C₄ alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

17. A method according to claim 16 wherein Q is selected from -CHR'-, -O- and -CO-NH-, where R' is hereinbefore defined.

18. A method according to any of claims 14 to 17 further comprising
5 separating and/or purifying the dye-labelled protein of interest by affinity chromatography.

19. A method according to any of claims 14 to 18 wherein said protein of interest is selected from antibody, antigen, protein, peptide, microbial
10 materials, cells and cell membranes.

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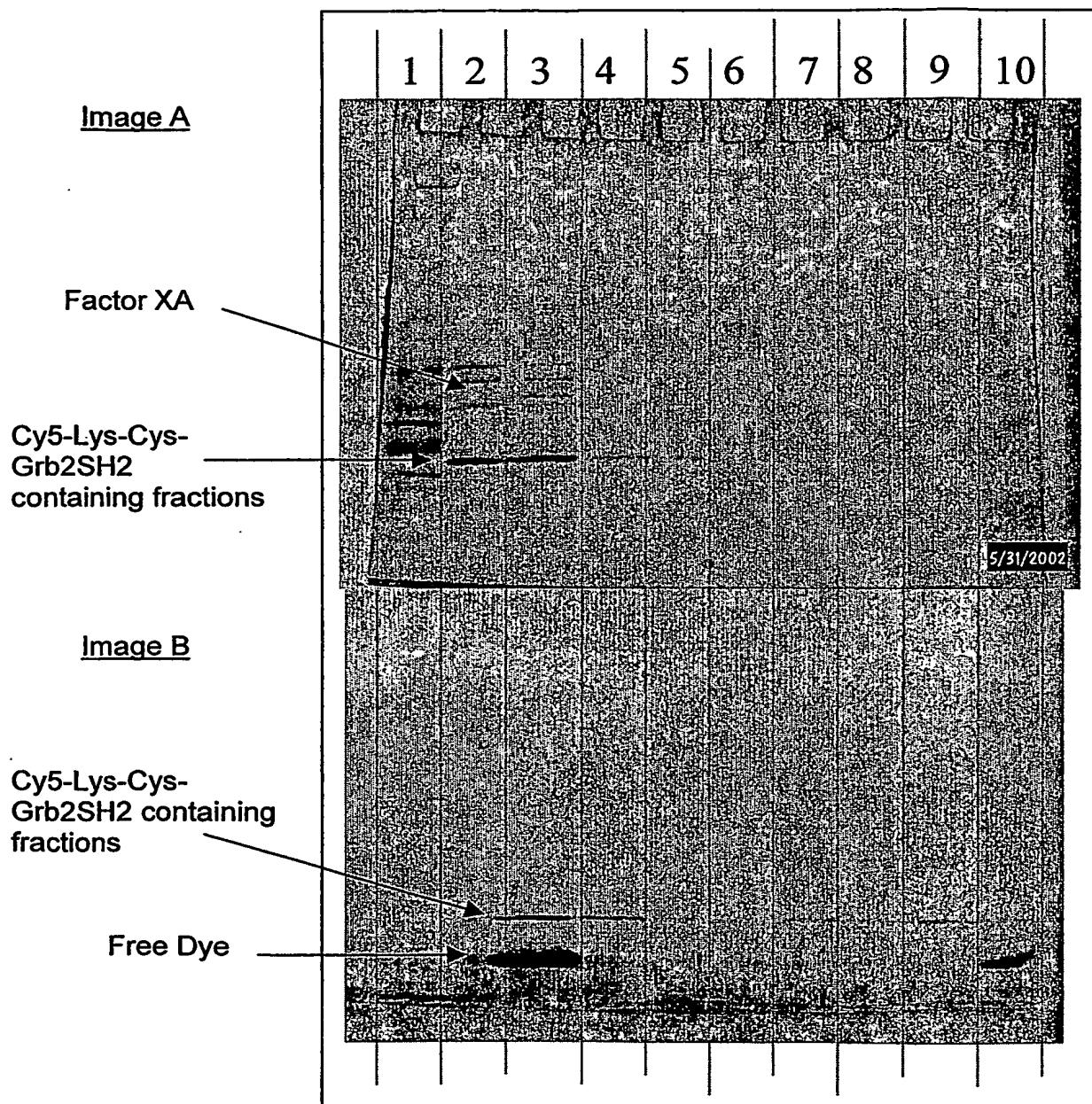
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Figure 1/1

SDS PAGE analysis - Coomassie blue stain and Typhoon Image



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 03/03196

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C09B23/08 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C09B G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHULER BENJAMIN ET AL: "Specific labeling of polypeptides at amino-terminal cysteine residues using Cy5-benzyl thioester" BIOCONJUGATE CHEMISTRY., vol. 13, no. 5, 18 July 2002 (2002-07-18), pages 1039-43, XP002259205 AMERICAN CHEMICAL SOCIETY, WASHINGTON., US ISSN: 1043-1802 cited in the application Abstract; Introduction page 1042, right-hand column, paragraph 2 -page 1043, left-hand column, paragraph 4 ---	1-19
A	US 5 569 587 A (WAGGONER ALAN S) 29 October 1996 (1996-10-29) cited in the application claims; examples ---	1-19 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

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Date of the actual completion of the international search

30 October 2003

Date of mailing of the international search report

10/11/2003

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US3 03/03196

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 64519 A (AMERSHAM PHARM BIOTECH UK LTD ;HATCHER MALCOLM J (GB); SCOTT BRIAN) 16 December 1999 (1999-12-16) & EP 1 086 179 B 23 April 2003 (2003-04-23) cited in the application -----	1-19

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 03/03196

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WO 9964519	A	16-12-1999		AT 238392 T AU 749437 B2 AU 4156899 A CA 2330974 A1 DE 69907185 D1 DK 1086179 T3 EP 1086179 A1 WO 9964519 A1	15-05-2003 27-06-2002 30-12-1999 16-12-1999 28-05-2003 11-08-2003 28-03-2001 16-12-1999